S100A1 MEDIATED SIGNALING IN THE NERVOUS SYSTEM

A Senior Scholars Thesis

by

ILKA MAZA

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

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Director for Honors and Undergraduate Research: Sumana Datta

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ABSTRACT

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S100 proteins are a large family of Ca^{2+} binding protein that have been implicated in the onset and progression of neurological diseases such as Alzheimer's disease (AD), cancer, and multiple sclerosis. However, the molecular mechanisms of altered S100 signaling in these diseases have not been elucidated. Recent studies from our laboratory demonstrate that one member of the family, S100A1, regulates amyloid precursor protein (APP) production and glycogen synthase kinase 3β (GSK3β) signaling in neuronal cell lines. In the brain, APP and GSK3β are involved in the development of senile plaques and tangles seen in AD and other dementias. However, neuronal cell lines do not faithfully mimic the diverse cell types/subtypes and complex anatomical organization of the intact brain. Therefore, this project uses a genetic approach to determine if S100A1 regulates amyloid precursor protein and GSK3β expression in the *in vivo* brain. $S100A1^{-/-}$ and wildtype levels for APP are 0.58 ± 0.10 vs. 0.46 ± 0.07 and for phospho-GSK3β are 0.22 ± 0.07 vs. 0.27 ± 0.06. However, these differences are not

statistically significant. Thus, our study demonstrates that S100A1 does not regulate the *in vivo* levels of APP or GSK3β.

DEDICATION

I would like to dedicate this thesis to my grandmother who has always supported me and has been diagnosed with Alzheimer's disease.

Also, this thesis is dedicated to my family who has motivated and encouraged me to pursue a career in science.

Lastly, I dedicate this thesis to everyone that is today suffering from Alzheimer's disease, whether directly or indirectly.

ACKNOWLEDGMENTS

It is a pleasure to thank those who made this thesis possible. Foremost, it is with immense gratitude that I would like to acknowledge the guidance, advice and support of Dr. Danna B. Zimmer who helped me develop an understanding of this subject.

I also would like to recognize the support and advice of David Mathai, Emily Roltsch, Katherine Albers and Bryce Gagliano.

NOMENCLATURE

Aβ	Amyloid Beta
AD	Alzheimer's Disease
APP	Amyloid Precursor Protein
$[Ca^{2+}]_i$	Intracellular Ca^{2+} Levels
EDTA	Ehtylene Diamine Triacetic Acid
GSK3β	Glycogen Synthase Kinase 3 Beta
HRP	Horseradish Peroxidase
PVDF	Polyvinylidene Difluoride
SERCA	Sarcoplasmic Endoplasmic Reticulum Calcium ATPase
TBS	Tris-buffered Saline

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Ca^{2+} signaling in the nervous system

All cells use Ca^{2+} as an intracellular second messenger. Cells maintain an intricate system of pumps and channels that maintain resting or basal Ca^{2+} levels in the nanomolar range. In response to external stimuli, intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) increase to the micromolar range. Dysregulation of Ca^{2+} homeostasis is a hallmark of many diseases including neurological disorders (Berridge et. al., 2010). In the nervous system, calcium ions activate fundamental processes such as release of neurotransmitters, axonal flow, long-term potentiation, cell motility, secretion, differentiation, and apoptosis. For example, depolarization in the presynaptic neuron causes voltage gated Ca^{2+} channels to open, increasing $[Ca^{2+}]_i$ levels which in turn triggers the release of neurotransmitters into the synaptic cleft. Changes in $[Ca^{2+}]_i$ are transduced into biological responses by Ca^{2+} binding proteins such as members of the S100/calmodulin/troponin superfamily. The long term goal of our research is to determine how S100A1, a member of the S100 family, transduces changes in the $[Ca^{2+}]_i$ into cellular responses and to develop agents that will inhibit the detrimental gain of S100A1 function that occurs in neurological diseases.

This thesis follows the style of Journal of Neuroscience.

The S100 protein family

The S100 proteins are a large family (>21 members) of Ca^{2+} binding proteins. These 10,000 molecular weight proteins were named S100 because of their solubility in 100% ammonium sulfate (Zimmer et al., 2005). S100 proteins have no enzymatic function and exert their biological effects by interacting with and modulating the activity of other proteins (target proteins) (Zimmer et al., 1998). All S100 proteins contain a unique non-canonical 14 amino acid EF-hand Ca^{2+} binding loop at the amino terminus (Zimmer et al., 2005). They also have a canonical 12 amino acid EF-hand Ca^{2+} binding loop at the carboxyl terminus that is found in all members of the S100/calmodulin/troponin superfamily (Figure 1) (Zimmer et al., 2005).

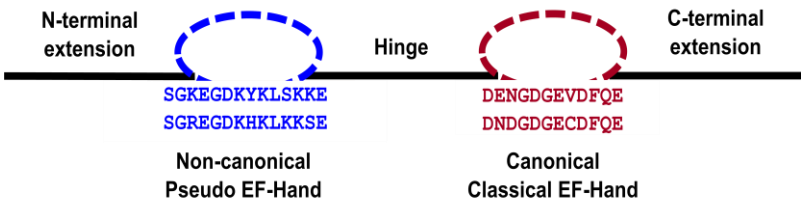

Figure 1. **S100 Ca^{2+} binding domains**. The loop on the left illustrates the non-canonical N-terminal EF hand Ca^{2+} binding domain that is unique to S100 family members with the amino acid sequence variations. In the middle we find a hinge region that connects both EF hands together. The loop on the right illustrates the canonical C-terminal EF-hand Ca^{2+} binding domain that is found in all members of the S100/calmodulin/troponin superfamily with the amino acid sequence variations.

Ca^{2+} binding to these motifs results in a conformational change which exposes the hydrophobic patch needed for target protein binding. S100 family members regulate a

large number of diverse cellular processes that include phosphorylation, transcription factors, Ca^{2+} homeostasis, cell growth and differentiation, and inflammation (Zimmer et. al., 1995). S100 family members have also been implicated in Alzheimer's disease, multiple sclerosis, Down's syndrome, epilepsy and cancer (Zimmer et. al., 2005).

S100A1 function

S100A1 is expressed in many tissues and cells. In the heart, genetic ablation of S100A1 impairs adaptation to hemodynamic stress, increases mortality in response to myocardial infarction, and results in hypertension (Desjardins et al., 2009). These effects have been attributed to S100A1 interaction with its target protein, the ryanodine receptor and SERCA pump (Desjardins et al., 2009). In skeletal muscle, S100A1 ablation delays and decreases action potentials (Prosser et al., 2010). The effects of S100A1 in skeletal muscle excitation-contraction coupling are due to S100A1 interaction with and modulation of the ryanodine receptor (Prosser et al., 2010). S100A1 is highly expressed in neuronal cells. Our lab was the first to report that S100A1 ablation in neuronal cells alters Ca^{2+} homeostasis, dendrite formation, microtubule polymerization, amyloid precursor protein (APP) expression and sensitivity to the neurotoxic Aβ peptide (Figure 2) (Zimmer et. al., 1998 and 2005). Thus, modulation of Ca^{2+} homeostasis by S100A1 appears to occur in all cells types.

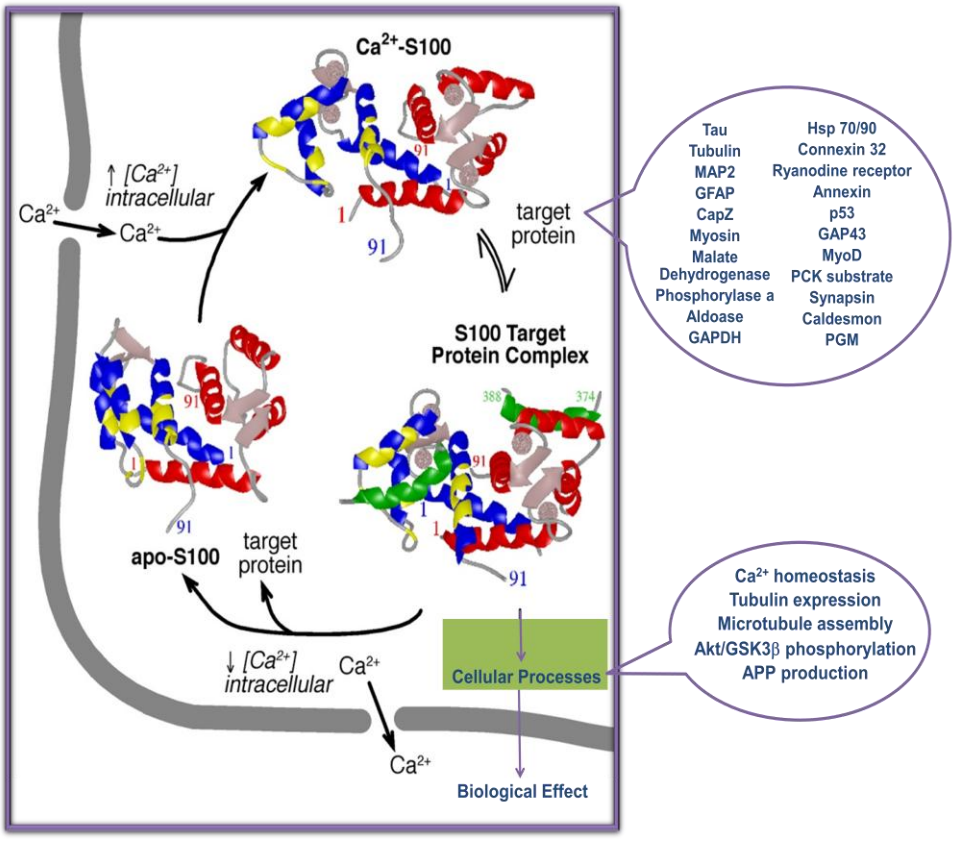

Figure 2. **S100A1 binds Ca^{2+} in neuronal cells.** Calcium enters the cell and binds to S100 protein. Hydrophobic patch of S100 is exposed and calcium bound S100 binds to the target protein, forming an S100 target protein complex. This complex can go on to carry out cellular processes and cause biological effects.

S100A1 regulation of APP

More recently, our group has shown that S100A1 ablation/inhibition decreases Alzheimer's disease pathology and increases cognitive function in an AD mouse model

(unpublished observations). The detrimental effects of S100A1 in AD may be due to increased APP expression. The APP gene is located on human chromosome 21q (Chow et. al., 2010). APP is also concentrated in synapses and has been implicated in synapse formation and repair (Chow et. al., 2010). In addition, it is up-regulated during cell differentiation and neuronal injury (Chow et. al., 2010). There are two pathways for the proteolytic processing of APP, non-amyloidogenic or amyloidogenic. In the normal brain, the non-amyloidogenic pathway predominates (Chow et. al., 2010). APP is sequentially cleaved by α- and γ-secretases, which does not result in the production of the neurotoxic Aβ peptide (Chow et. al., 2010). In the AD brain, the amyloidogenic pathway predominates (Chow et. al., 2010). APP is sequentially cleaved by β- and γ-secretases which results in Aβ peptide production (Chow et. al., 2010). The Aβ peptide aggregates and forms plaques, which are a pathological hallmark of AD (Chow et. al., 2010). The beneficial effects of S100A1 ablation/inhibition on AD pathology are predicted to be attributable to decreases in APP/Aβ levels.

S100A1 regulation of GSK3β

Previous studies from our laboratory demonstrate that S100A1 signaling regulates glycogen synthase kinase-3β (GSK3β). GSK3β is responsible for regulating neurogenesis, axonal growth, and tau phosphorylation in the normal brain (Hur and Zhou, 2010). In the AD brain, GSK3β hyperphosphorylates the tau protein, resulting in the neurofibrillary tangles seen in AD patients (Hernández et al., 2010). GSK3β is

inactivated by AKT phosphorylation (Liang et al., 2003). Thus we predict that ablation/inhibition of S100A1 will increase phospho-GSK3β levels in the *in vivo* brain.

Project description

This project tests our central hypothesis that S100A1 regulates APP and phospho-GSK3β levels in the *in vivo* brain. Immunoblotting was used to compare APP and phospho- GSK3β levels in S100A1 knockout mice and wildtype littermate controls. Based on our previous *in vitro* studies, we expect that S100A1 knockout mice will exhibit a 30 percent reduction in APP levels and a 3-fold increase in phospho-GSK3β levels (Zimmer et. al., 2005). Collectively, these results will identify S100A1 as a therapeutic target for Alzheimer's disease and other neurological disorders in which increased APP expression contributes to the disease process.

CHAPTER II

METHODS

Sample acquisition/processing

Total brain lysates were prepared from S100A1 knockout (experimental) and wildtype littermates (control) mice by homogenizing 0.05 g wt weight per 1 mL of TPER containing 10 mM EDTA and 3X HALT inhibitor (Thermo Scientific, Rockford, IL). Homogenates were separated into soluble and insoluble fractions by centrifugation at 14000 rpm for 1 minute at 4°C. Supernatant was collected; aliquots were prepared, and stored at -80°C. Protein concentrations were determined by Bradford assay as previously described (Bradford, 1976).

Immunoblotting

Ten µg total protein in 4% (w/v) SDS, 0.075 glycerol, 1.2 mg/mL Bromophenol blue,1 mM DTT, 40 mM Tris-HCL, pH 6.8 (Sample Buffer) was sized fractionated using a 4-20% SDS-polyacrilamide gel(Bio-Rad, Hercules, CA). Precision Plus Protein WesternC Standard was used as size markers (Bio-Rad). Gels were transferred to a nitrocellulose membrane (Millipore, Billerica, MA) for 60 minutes at 30 Volts in a Bio-Rad Mini-Protean Tetra System with 20% methanol in 0.096M Glycine and 0.0125 M Tris; pH 8.3 (transfer buffer) and a refrigerant pack. Blots were blocked in 5% (w/v) non-fat dry milk, 0.1% (v/v) Tween 20, 200 mM NaCl, 50 mM tris, pH 7.5, Tris-buffered saline (TBS) (Non-fat Dry Milk TBST) for 60 min at room temperature.

Detection of proteins was performed by incubating in primary antibody for one hour at room temperature or overnight at 4°C. Primary antibodies included, a mouse monoclonal C-terminal Anti-APP antibody (1-500 dilution of, A8717, Sigma-Aldrich) that detects full length APP, a rabbit polyclonal Phospho-GSK3β antibody (1-250 dilution of, #9336, Cell Signaling Technology), a rabbit monoclonal GSK3β antibody (1-500 dilution of, # 9315, Cell Signaling Technology). After rinsing three times for five minutes in 20mM Tris, pH 8.0, 150 mM NaCl, and 0.05% Tween-20 (TBST), blots were incubated in a species-specific secondary antibody containing 1µL Streptactin HRP for one hour at room temperature. Secondary antibodies included HRP-labeled Goat anti-rabbit antibody (1:10,000; Bethyl, Montgomery, TX), and HRP-labeled Goat anti-mouse IgG (1:15,000, KPL) in non-fat dry milk and Precision Protein StrepTactin-HRP conjugate (Bio-Rad). After washing in TBST, antibody binding was visualized with a Super Signal West Femto Maximum Sensitivity Substrate (Thermo Scientific).

For quantification, membranes were imaged using the ImageQuant LAS 4000 mini system. ImageQuant TL 7.0 software was used to quantify pixels in bands. To minimize variability and loading error, β-actin was used to normalize measured band pixels. In the case of C-terminal antibody, the blot was cut between 50-75 kDal; the lower half was incubated in a mouse monoclonal β-actin antibody (1-3,000 dilution of, A1978, Sigma-Aldrich), and HRP-labeled Goat anti-mouse IgG (1:15,000 dilution of, A90-116P, Bethyl Laboratories), while the upper half was incubated in C-terminal antibody. Normalization consisted of dividing the pixel area for each APP band by the pixel area

for each β-actin band for each blot. The measured pixel area of all S100A1 knockout blots after normalization was averaged as well as the wildtype blots. For phospho-GSK3β, blots were normalized to total GSK3β. Phospho-GSK3β was then divided by the total GSK3β as previously described for C-terminal APP. All data were expressed as the mean relative levels $\pm$ SEM (n=4 (phospho-GSK3β) n=6 (APP) for S100A1$^{-/-}$ and wildtype). A t-test was used to determine the significance ($p < 0.05$) of difference between samples groups.

CHAPTER III

RESULTS

Quantification of APP levels

Given the importance of APP in neurodegenerative diseases such as AD, we sought to determine if S100A1 regulated APP levels in the cortex. A western blotting procedure was used to quantify APP levels in the cortex of S100A1$^{-/-}$ and wildtype mice. First, a comparison of Amersham Basic, Amersham Plus, Amersham Advanced, West Pico, and West Femto chemiluminescent detection kits was performed. Serial dilutions of an HRP conjugated secondary antibody were immobilized on Amersham nitrocellulose and PVDF; and then detected with the various chemiluminescence reagents. The West Femto Kit and the nitrocellulose membrane exhibited the greatest sensitivity. Next, we compared two primary antibodies that detect full length APP: 22C11 and C-terminal antibody. Interestingly, the 22C11 antibody did not detect full length APP (~120 kDal). However, the C-terminal antibody did detect full length APP band (~120 kDal). A comparison of 1-500, 1-750, 1-2000, and 1-5000 dilution of C-terminal antibody indicated that 1-750 dilution was optimal. To minimize loading errors, β-actin was used to normalize measured levels of APP. Two β-actin antibodies were compared and optimized. A β-actin antibody from Bethyl laboratories did not detect the predicted 42 kDal band. However, a β-actin antibody (1-3,000 dilution) from Sigma laboratories did recognize the 42 kDal β-actin band.

Following optimization, levels of full length APP were quantified in brain extracts from S100A1$^{-/-}$ and wildtype control mice. Data were expressed as the mean relative APP levels ± SEM (n=6). A Student T-test was assessed to determine the significance ($p<0.05$) of measured differences. S100A1$^{-/-}$ and wildtype levels for APP are 0.58 ± 0.10 vs. 0.46 ± 0.07 (Figure 3). However, these differences are not statistically significant.

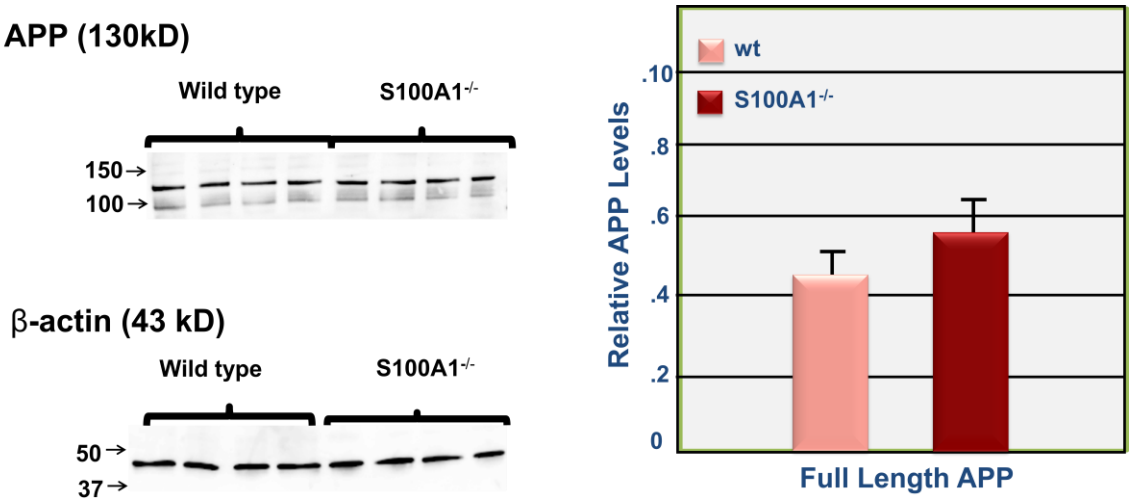

Figure 3. **Full length APP levels in S100A1$^{-/-}$ and wildtype mice.** Representative APP and β-actin immunoblots are shown in the left-hand panel. The histograms on the right contain the mean APP levels ± SEM (n=6) in S100A1$^{-/-}$ and wildtype littermates normalized to β-actin levels. Although APP levels were higher in S100A1$^{-/-}$ mice, this difference was not statistically significant.

Phospho-GSK3β levels do not respond to S100A1 genetic ablation

GSK3β hyperphosphorylates the microtubule-associated tau protein resulting in the neurofibrillary tangles seen in AD patients. Interestingly, from previous studies we know that S100A1 regulates AKT, a protein kinase that phosphorylates and inactivates GSK3β (Zimmer et al., 2005). Thus, we hypothesize that S100A1 ablation will increase phospho-GSK3β levels *in vivo*. In order to quantify levels of phospho-GSK3β we had to optimize the conditions. The chemiluminescence detection kit and membrane is same as previously mentioned. We also determined that lysates must contain phosphatase inhibitors for phospho-GSK3β (~ 46 kDal) detection. A comparison of 1-250, 1-500, and 1-1000 dilution of phospho-GSK3β antibody indicated that 1-250 dilution was optimal. Total GSK3β (46 kDal) levels were used for normalization and a 1-250 dilution of primary antibody was optimal. Next, we measured and quantified amounts of phospho-GSK3β in S100A1$^{-/-}$and wildtype mice. Data were expressed as the mean relative phospho-GSK3β levels $\pm$ SEM (n=4). A Student T-test was assessed to determine the significance ($p<0.05$) of measured differences. S100A1$^{-/-}$ and wildtype levels for phospho-GSK3β are 0.22 ± 0.07 vs. $0.27 \pm .06$ (Figure 4). However, this difference was not statistically significant.

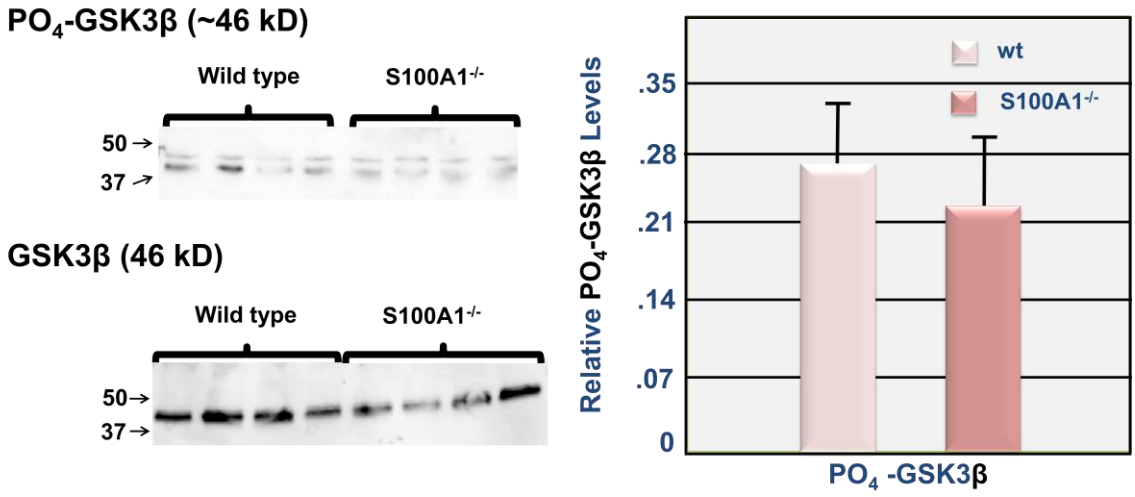

Figure 4. **Phospho-GSK3β levels in S100A1$^{-/-}$ and wildtype mice.** Representative phospho-GSK3β and GSK3β immunoblots are shown in the left-hand panel. The histograms on the right contain the mean phospho-GSK3β levels ± SEM (n=4) in S100A1$^{-/-}$ and wildtype littermates normalized to total GSK3β levels. Although phospho-GSK3β were lower in S100A1$^{-/-}$ mice, this difference was not statistically significant**.**

CHAPTER IV

SUMMARY AND CONCLUSION

From this study we concluded that S100A1 does not regulate APP production or GSK3β signaling despite previous *in vitro* studies. Previous *in vitro* studies from our laboratory demonstrate that S100A1 ablation causes a 30% reduction in APP levels and a 3-fold increase in phospho-GSK3β levels. These differing results emphasize the importance of studying S100A1function in *in vivo* systems. These discrepancies may be due to regional variation in S100A1 function. S100A1 is highly expressed in areas of the brain such as the olfactory bulb and the cerebellum (unpublished observations) and the cortex, which was used in this study, expresses low levels. Thus, in future studies S100A1 function in brain regions with higher expression must be examined. Another explanation is that, whole brain homogenates may mask any changes that occur in specific cell populations.

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